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A Microwell Cytotoxicity Assay using *Artemia salina* (Brine Shrimp)

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Abstract

A new microplate assay for cytotoxicity testing using *A. salina* has been developed and shown to give results comparable to a previously published test-tube method. The assay reliably detected all of the compounds toxic to KB cells in a series of 21 pharmacologically active agents, except for two which require metabolic activation in man. Four quassinoids with cytotoxic and antiplasmodial activity were also toxic to the brine shrimp while quassin itself was inactive in all three systems. It is proposed that this assay provides a convenient means by which the presence of cytotoxic quassinoids may be detected during the fractionation of plant extracts.

Key words

Artemia salina, brine shrimp, cytotoxicity, bioassay, *Plasmodium falciparum*, KB cells, quassinoids.

Introduction

Brine shrimp larvae (nauplii) have been used as a bioassay for a variety of toxic substances and this method has also been applied to plant extracts in order to facilitate the isolation of biologically active compounds (1–3). This methodology requires relatively large quantities of material (20 mg for crude extracts and 4 mg for pure compounds) and the preparation of dilutions is time-consuming thus limiting the number of samples and dilutions that can be tested in one experiment. In this paper we report the use of a convenient microplate assay which overcomes the above disadvantages.

Materials and Methods

Brine shrimp eggs (*Artemia salina*) obtained locally (Interpet Ltd. Dorking, England) were hatched in artificial sea water prepared from sea salt (Sigma Chemical Co., U.K.) 40 g/l supplemented with 6 mg/l dried yeast and oxygenated with an aquarium pump. After 48 hours incubation in a warm room (22–29 °C), nauplii were collected with a pasteur pipette after attracting the organisms to one side of the vessel with a light source. Nauplii were separated from the eggs by pipetting them 2–3 times in small beakers containing sea water.

Test compounds

Bruceines A, C, and D and brusatol were isolated as previously described (4). Villastonine was isolated from *Alstonia angustifolia* as previously reported (5). Homoharringtonine, isoharringtonine, and cephalotaxine were kindly supplied by Dr. Powell, United States Department of Agriculture, Illinois, and quassin was a gift from Bush, Boake and Allen Ltd. U.K., thymol was obtained from May and Baker Ltd, U.K., actinomycin D, from Aldrich Chemical Co. U.K., and atropine sulphate, chloramphenicol, quinidine sulphate and quinine hydrochloride were supplied by BDH, U.K. All other compounds were obtained from Sigma Chemical Co. U.K.

Test methodology

Samples for testing (0.6 mg) were made up to 1 mg/ml in artificial sea water except for water insoluble compounds which were dissolved in 50 µl DMSO prior to adding sea water.

Serial dilutions were made in the wells of 96-well microplates (ICN Flow, U.K.) in triplicate in 100 µl sea water. Control wells with DMSO were included in each experiment. A suspension of nauplii containing 10–15 organisms (100 µl) was added to each well and the covered plate incubated at 22–29 °C for 24 hours. Plates were then examined under a binocular microscope (× 12.5) and the numbers of dead (non-motile) nauplii in each well were counted. 100 µl methanol were then added to each well and after 15 minutes the total numbers of shrimp in each well were counted. LC_{50} values were then calculated by Probit analysis (6).

Results and Discussion

The results obtained with a number of pharmacologically active compounds, some of which are cytotoxic, are compared with results reported using a previous method in which test tubes were used (7); with the exception of thymol, LC_{50} values determined by the two methods are similar. However, when thymol was tested in our laboratory using the test tube method an LC_{50} value of 46.7 ± 12.0 µM was obtained which is in close agreement with that found using the new microplate test.

The test was predictive of cytotoxicity for all the compounds listed in Table **1** except for cyclophosphamide which is known to require metabolic acti-

Table 1 Comparison of the values obtained in the brine shrimp microtechnique with previously reported data.

Drugs	Micro-Technique LC_{50} (μM) ± sd		Reported Values μM
Anisomicin	35.2 ± 14.2	(2)[a]	–
Atropine sulphate	> 1000	(3)	987.3[b]
Berberine chloride	25.2 ± 2.9	(3)	60.5[b]
Caffeine	> 1000	(3)	> 1000[b]
Cephalotaxine	549.9 ± 83.7	(3)	–
Cyclophosphamide	> 1000	(3)	–
Ephedrine	> 1000	(3)	> 1000[b]
Homoharringtonine	7.4 ± 4.7	(3)	7.5[c]
Isoharringtonine	13.9 ± 6.2	(2)	–
Nicotine	> 1000	(2)	> 1000[c]
Podophyllotoxin	15.0 ± 2.1	(2)	5.8[b]
Quinidine sulphate	518.8 ± 126.9	(3)	287.8[b]
Thymol	56.3 ± 10.6	(3)	> 1000[b]; 46.7 ± 12.0[d]

[a] Indicates number of experiments performed.
[b] Data obtained from Meyer et al. (7).
[c] Data obtained from Anderson et al. (2).
The previously reported data were transformed to μM for comparison purpose only.
[d] Value obtained in our laboratory using the test tube method.

Table 2 Comparison of brine shrimp toxicity against *in vitro* KB cell cytotoxicity of compounds with different mechanisms of action.

Drugs	Brine shrimps LC_{50} (μM) ± sd		KB Cell ED_{50} (μM)
Actinomycin D	$3.8 \times 10^{-4} \pm 1.4 \times 10^{-4}$	(2)[a]	0.012[b]
Chloramphenicol	> 1000	(2)	> 50[b]
Chloroquine diphosphate	> 1000	(2)	153[c]
Cycloheximide	104.6 ± 1.0	(3)	3.31[b]
Emetine HCl	29.0 ± 6.8	(4)	0.673[b]
6-Mercaptopurine	> 1000	(2)	3.42[b]
Podophyllotoxin	15.0 ± 2.1	(2)	0.007[c]
Quinine	> 1000	(2)	333[b]
Villastonine	10.8 ± 4.8	(3)	11.6[d]

[a] Indicates number of experiments performed.
[b] Data obtained from Anderson (13).
[c] Data obtained from Anderson et al. (14).
[d] Data obtained from Wright et al. (5).

Table 3 Activities of quassinoids against brine shrimps, KB cell and *P. falciparum in vitro*.

Quassinoid	Brine shrimp LC_{50} (μM) ± sd		KB Cell ED_{50} (μM)[b]	*P. falciparum* IC_{50} μM
Bruceine A	0.887 ± 0.24	(2)[a]	0.188	0.021[c]
Bruceine C	0.798 ± 0.17	(2)	0.037	0.009[c]
Bruceine D	4.47 ± 0.19	(2)	2.82	0.037[c]
Brusatol	1.69 ± 0.137	(2)	0.196	0.006[c]
Quassin	> 1000		> 50	247[d]

[a] Indicates number of tests performed.
[b] Data obtained from Anderson et al. (15).
[c] O'Neill et al. (4).
[d] Data obtained from Anderson (13).

vation in the liver for activity in man. Since *A. salina* does not possess the enzymes required for this process, cyclophosphamide would not be expected to be toxic to this organism.

Table 2 shows the LC_{50} values determined for a number of biologically active compounds which have various modes of action together with their reported ED_{50} values against KB cells (human nasopharyngeal carcinoma). Of the six compounds active against KB cells, five were also toxic to nauplii. However, there was little correlation observed in the degree of toxicities seen between the two methods. Emetine was more toxic to KB cells than villastonine but against the brine shrimp the converse was found to be the case. Similarly, although podophyllotoxin and actinomycin D were equitoxic to KB cells, the brine shrimp was > 50,000 times more sensitive to the latter than to the former. One cytotoxic compound, 6-mercaptopurine was not toxic to *A. salina* and this is explicable as its purine metabolism is markely different from that in mammalian cells (8) and it is possible that the intracellular activation which occurs in man may not take place in *A. salina*.

Quassinoids are constituents of the Simaroubaceae and have potent *in vitro* activity against *P. falciparum* (4). These compounds inhibit protein synthesis in both mammalian cells and malaria parasites (9), and, as shown in Table 3, were found to be toxic to the brine shrimp. Quassin which does not possess cytotoxic or antiplasmodial activity was, as expected, devoid of toxicity. These results suggest that for this group of compounds the brine shrimp test is suitable for the screening of plant extracts and fractionation of cytotoxic quassinoids, thus obviating the need for the difficult and expensive antiplasmodial test at every stage in the isolation procedure. Similarly, the brine shrimp test has been used as a convenient assay for the detection of antifilarial activity of avermectin analogs (10).

This approach may be useful for other types of biologically active compounds where the brine shrimp responds similarly to the corresponding mammalian systems. For example, the DNA-dependent RNA polymerases of *A. salina* have been shown to be similar to the mammalian type (11) and the organism has an ouabaine-sensitive Na^+ and K^+ dependent ATPase (12), so that compounds or extracts acting on these systems would be expected to be detected in this assay.

Conclusion

The method described above provides a simple and inexpensive screening test for cytotoxic compounds. It has the advantages of requiring only small amounts (0.6 mg) of compounds and the employment of microplate technology facilitates the testing of large number of samples and dilutions. The use of methanol to kill live shrimps at the end of the experiment enables rapid counting of the total numbers of shrimps. Although the assay did not detect those compounds which require metabolic activation in man, it may be conveniently used where, as in the case of the quassinoids, the brine shrimp is a reliable detector of biological activity.

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